Review

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Epigenetic aberrations in human pluripotent stem cells

Shiran Bar & Nissim Benvenisty*

Abstract

Human pluripotent stem cells (hPSCs) are being increasingly utilized worldwide in investigating human development, and modeling and discovering therapies for a wide range of diseases as well as a source for cellular therapy. Yet, since the first isolation of human embryonic stem cells (hESCs) 20 years ago, followed by the successful reprogramming of human-induced pluripotent stem cells (hiPSCs) 10 years later, various studies shed light on abnormalities that sometimes accumulate in these cells *in vitro*. Whereas genetic aberrations are well documented, epigenetic alterations are not as thoroughly discussed. In this review, we highlight frequent epigenetic aberrations found in hPSCs, including alterations in DNA methylation patterns, parental imprinting, and X chromosome inactivation. We discuss the potential origins of these abnormalities in hESCs and hiPSCs, survey the different methods for detecting them, and elaborate on their potential consequences for the different utilities of hPSCs.

Keywords DNA methylation; epigenetic alterations; human pluripotent stem cells; imprinting; X chromosome inactivation

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Introduction

Human pluripotent stem cells (hPSCs) represent the *in vitro* counterparts of embryonic cells in human pre-implantation development. Their ability to self-renew and differentiate into all three germ layers in culture establishes them as highly valuable in research of early human embryogenesis and disease modeling as well as a promising source for regenerative therapy (Ben-David *et al*, 2012).

Apart from their abundant potential, the unique properties of hPSCs also contribute to their tumorigenicity and genomic instability. Injecting hPSCs under the skin of immunodeficient mice leads to the formation of benign teratomas, which are comprised of differentiated cells of all three germ layers. Yet, following extended growth in culture, these cells may undergo selection for altered karyotypes, which can subsequently give rise to aggressive tumors. The prevalence and consequences of genetic aberrations in hPSCs are the subject of extensive discussions (Ben-David & Benvenisty, 2011). These modifications include chromosomal abnormalities, copy number variations, and point mutations which are repeatedly selected during hPSC propagation. Whereas variations in *TP53* were recently found to significantly dominate the landscape of point mutations in hPSCs (Merkle *et al*, 2017), the most common chromosomal abnormalities in hPSCs include full or partial trisomies of chromosomes 1, 8, 12, 17, and X, and duplications of 20q11.21 (Lund *et al*, 2012; Weissbein *et al*, 2014). As similarly observed in a variety of tumors, these increased copy number variations are thought to confer selective advantage of cultured cells by upregulating growth-promoting genes. Notwithstanding, in addition to such genetic changes, epigenetic aberrations are also inherently capable of affecting the dosage of gene expression. Markedly, epigenetic changes affect cellular phenotypes and emerge extensively in culture, but although they are inherited through mitosis, they are not associated with changes in genomic sequences. Epigenetic abnormalities include deviations in DNA methylation, histone modifications, and other inherited chromatin marks, which drive changes in gene expression and cellular integrity (Flavahan *et al*, 2017).

In this review, we focus on three epigenetic abnormalities, which were most studied in hPSCs and include DNA methylation alterations, loss of parental imprinting, and variation in X chromosome inactivation. These aberrations significantly influence transcription of individual genes, up to an entire chromosome. We discuss the forces driving these aberrations (including when and how they might arise), outline different methods to detect them, and examine their consequences for applications of hPSCs.

Human pluripotent stem cell types

hPSCs can be classified by the method used to derive them. Human embryonic stem cells (hESCs) were the first hPSCs to be successfully propagated *in vitro* and they are isolated from the inner cell mass (ICM) of *in vitro* fertilized embryos. Yet, this requirement for human blastocysts bears ethical concerns and limits the capacity to generate profuse and diverse hESC lines. Therefore, the generation of human-induced pluripotent stem cells (hiPSCs) by direct reprogramming from somatic cells was a promising advancement and enabled more laboratories to produce new cell lines and join the field (Hochedlinger & Jaenisch, 2015). Moreover, isolating hiPSCs from

Department of Genetics, The Azrieli Center for Stem Cells and Genetic Research, Silberman Institute of Life Sciences, The Hebrew University, Jerusalem, Israel
*Corresponding author. Tel: +972 2 6586774; E-mail: nissimb@mail.huji.ac.il

disease patients potentially adds a significant progress toward transplantations of autologous differentiated cells. More recently, reprogramming was also achieved by introducing a somatic nucleus into an enucleated oocyte to generate human somatic cell nuclear transfer PSCs (SCNT-PSCs; Tachibana *et al*, 2013; Yamada *et al*, 2014).

Although hPSCs that are grown in standard culture conditions mimic most functional properties of ICM in differentiation, they significantly diverge in their epigenetic and transcriptional landscapes. These dissimilarities prompted the search for culture conditions that will reset and maintain hPSCs in a "naïve" state, which presumably better resemble pluripotent cells *in vivo*. Consequently, there are currently several different protocols for isolating and resetting hPSCs to become naive (Sagi & Benvenisty, 2016; Bates & Silva, 2017; Collier & Rugg-Gunn, 2018).

In this review, we will discuss and compare epigenetic aberrations in the different types of hPSCs, including hESCs, hiPSCs, SCNT-PSCs, and naïve hPSCs.

Types of epigenetic aberrations in hPSCs, their origins, and detection methods

DNA methylation patterns

The most extensively studied epigenetic modification is DNA methylation, in which methyl groups are added to the fifth carbon of cytosine residues, thus forming 5-methylcytosine (5-mC), mainly in the context of CpG dinucleotides. In mammals, 5-mC is prevalent in diverse genomic regions, including transposable elements, imprinted regions, and gene bodies, as well as in some inactive promoters, whereas CpG-rich regions are mostly devoid of methylation (Suzuki & Bird, 2008). This modification is maintained during replication and is considered relatively stable in somatic cells, yet various reports also emphasize its dynamic regulation during development (Hackett & Surani, 2013; Messerschmidt *et al*, 2014), its sensitivity to aging and environmental forces (Jung & Pfeifer, 2015; Mitchell *et al*, 2016), and high frequency of alterations in many cancers (Flavahan *et al*, 2017). Although DNA methylation is mostly associated with gene repression, its regulatory function is emerging as more complex and dependent on CpG density. Thus, in many instances, 5-mC is apparently secondary to other repressive marks and contributes to sustaining heterochromatic memory and long-term gene silencing. Accordingly, the absence of methylation at a promoter does not necessarily prompt the activation of its cognate gene, as additional factors are required for transcription initiation (Hackett & Surani, 2013). Nevertheless, the function of 5-mC is critical in silencing transposons and also in disrupting their sequence over time by favoring mutations through deamination. These roles were suggested to be the drivers for 5-mC selection during evolution (Yoder *et al*, 1997).

After fertilization, DNA methylation is globally erased, reaches a minimum in the blastocyst stage, and is then re-established during differentiation (Iurlaro *et al*, 2017). Although human ICM cells exhibit very low levels of genome-wide DNA methylation, hPSCs are globally hypermethylated even when compared with somatic cells (Nishino & Umezawa, 2016). Moreover, the global persistence of DNA methylation is essential for hPSC survival, as knockout of DNA methyltransferase 1 (*DNMT1*), which is responsible for catalyzing the addition of 5-mC on the newly replicated strand during S phase, results in rapid cell death (Liao *et al*, 2015). Similar consequences are observed following treatment with the chemical demethylating agent 5-aza-2′-deoxycytidine (5-azadc; Bar-Nur *et al*, 2012). The necessity for *DNMT1* in hPSCs is in complete contrast to mouse PSCs, which are resistant to simultaneous knockout of *Dnmt1*, *Dnmt3a*, and *Dnmt3b* (Tsumura *et al*, 2006; Liao *et al*, 2015). The significant differences in DNA methylation between human blastocysts and hPSCs, along with discrepancies in gene expression, are a significant driver in the research of naive hPSCs. Therefore, naive hPSCs should exhibit similar epigenetic features to that of ICM, including global hypomethylation (Theunissen *et al*, 2016) and resistance to *DNMT1* downregulation.

DNA methylation aberrations have been observed in various hPSC lines (Lund *et al*, 2012). These include 5-mC variations in gene promoters (Fig 1) and non-coding regions, as well as residual DNA methylation signatures from somatic cells in iPSCs. Accumulating evidence illustrates similarities between gene-specific DNA methylation aberrations in hPSCs and those prevalent in malignancies (Planello *et al*, 2014; Konki *et al*, 2016; Weissbein *et al*, 2017). The generation of more accurate and comprehensive maps of whole-genome methylation should promote additional analyses on gene-specific 5-mC aberrations in hPSCs, which may enhance our understanding of their implications and elaborate on the epigenetic dynamics in these cells.

Origins of DNA methylation aberrations

Analyzing DNA methylation in multiple hPSC lines identified characteristic patterns that distinguish them from somatic cells (Bibikova *et al*, 2006; Deng *et al*, 2009), including abnormal hypermethylation of tumor suppressor genes (Calvanese *et al*, 2008). During growth in culture, it has been shown that these cells acquire variable DNA methylation aberrations in multiple sites (Bock *et al*, 2011; Nazor *et al*, 2012), many of which also appear in tumors (International Stem Cell Initiative *et al*, 2011). These culture-induced aberrations are mostly stable and persist throughout differentiation (Allegrucci *et al*, 2007; Nazor *et al*, 2012).

Using genome-wide methylation maps of hPSCs as a resource, a separate study concentrated on recurrent gene-specific alterations and detected several genes that repeatedly gain methylation in hPSCs of higher culture passage, and whose expression was silenced. Subsequently, this study focused on the gene *TSPYL5*, which was also implicated in several tumors, demonstrating that its deletion in cells in which it was still active, resulted in overexpression of pluripotency and growth-related genes as well as downregulation of tumor suppressors and genes associated with differentiation (Weissbein *et al*, 2017). Additional studies identified frequent hypermethylation and reduced expression of the antioxidant gene catalase (*CAT*; Bock *et al*, 2011), occurring specifically in hPSCs featuring an abnormal karyotype (Konki *et al*, 2016). Particularly, hPSC lines, which are prone to chromosomal instability, undergo a gradual methylation increase near the start site of this gene in normal and abnormal cells, suggesting that DNA methylation aberrations in a gene involved in reducing oxidative stress and DNA damage may predispose these cells toward the accumulation of genetic aberrations. Correspondingly, the authors also found the same aberrations in embryonal carcinoma cell lines and downregulation of *CAT* in several other cancers (Konki *et al*, 2016).

Interestingly, the majority of reported aberrations in hPSCs involve gaining of methylation accompanied by gene silencing

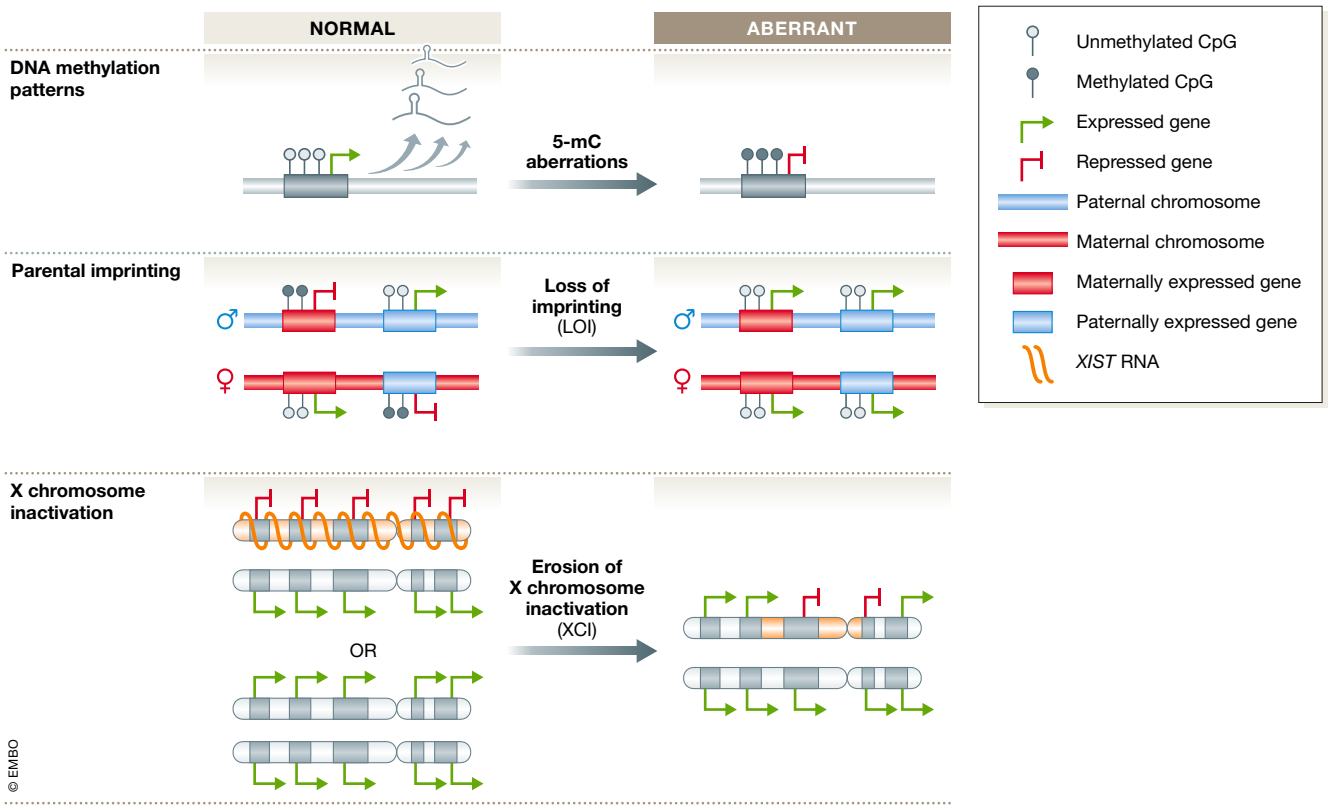

Figure 1. Types of epigenetic aberrations in hPSCs.

Top: DNA methylation aberrations in hPSCs are mainly caused by promoter hypermethylation and lead to gene silencing. Center: In normal hPSCs, imprinted genes are expressed from either the paternal or maternal allele. Loss of imprinting involves hypomethylation of imprinted DMRs, driving aberrant biallelic expression of imprinted genes. Bottom: Female cells in early pre-implantation development have two active X chromosomes. Later in development, they initiate XCI, which is achieved by *XIST* coating and results in silencing one X chromosome randomly. Many hPSCs exhibit aberrant erosion of XCI, which is characterized by *XIST* repression and partial reactivation of genes from the silent X chromosome.

(Fig 1). Such hypermethylation requires the alteration of both alleles, whereas gene activation is potentially feasible following hypomethylation of a single allele. A possible explanation for this potential discrepancy may be that loss of methylation of a growth-promoting gene might not be sufficient for its activation. Alternatively, this could also be attributed to the global tendency of hPSCs toward hypermethylation, or to a technical limitation of studies utilizing methylation arrays, which are mostly enriched in non-methylated CpG islands. Overall, these studies signify that gene-specific methylation aberrations, which are also present in cancers, are strongly selected during hPSC growth *in vitro* (Fig 2). As these selection forces are apparently similar to those driving genetic aberrations, previous recommendations for improving cell-culture practices aimed at minimizing cellular stress (Weissbein *et al*, 2014) could also be beneficial for reducing the selection toward DNA methylation abnormalities in hPSC. Still, since epigenetic modifications are also directly affected by the environment, further research aimed at finding methods to amend culture conditions to support the stability of DNA methylation is necessary.

In addition to their emergence over time in culture, DNA methylation aberrations were also observed to be highly induced upon reprogramming of human somatic cells to iPSCs (Fig 2). This process encompasses substantial epigenetic changes, transforming the chromatin landscape of a differentiated cell to that of an undifferentiated one by inducing the expression of key pluripotency genes, thus leading to broad changes in the overall transcription pattern. Accordingly, the efficiency of reprogramming was found to be inversely correlated with the extent of differences in CpG methylation between the somatic cell-of-origin and hPSCs (Ruiz *et al*, 2012). Correspondingly, many reported hiPSC lines were insufficient in completely erasing their somatic identity, thus carrying residual methylation of their source cells in various regions. This resulted in an epigenetic memory at distinct regions which bear differential methylation between hESCs and hiPSCs (Kim *et al*, 2010, 2011; Bar-Nur *et al*, 2011; Lister *et al*, 2011; Ohi *et al*, 2011; Roost *et al*, 2017). hiPSCs were also shown to maintain somatic non-CG methylation, especially near centromeres and telomeres in regions marked by H3K9me3 (Lister *et al*, 2011). Nevertheless, reprogramming mouse and human somatic cells by nuclear transfer facilitated a less aberrant methylation pattern which is similar to that of ESCs (Kim *et al*, 2010; Ma *et al*, 2014). Additional methylation alterations, which are not found in either the normal somatic source cells or hESCs, are acquired during reprogramming and vary between different hiPSC lines (Doi *et al*, 2009; Bock *et al*, 2011; Lister *et al*,

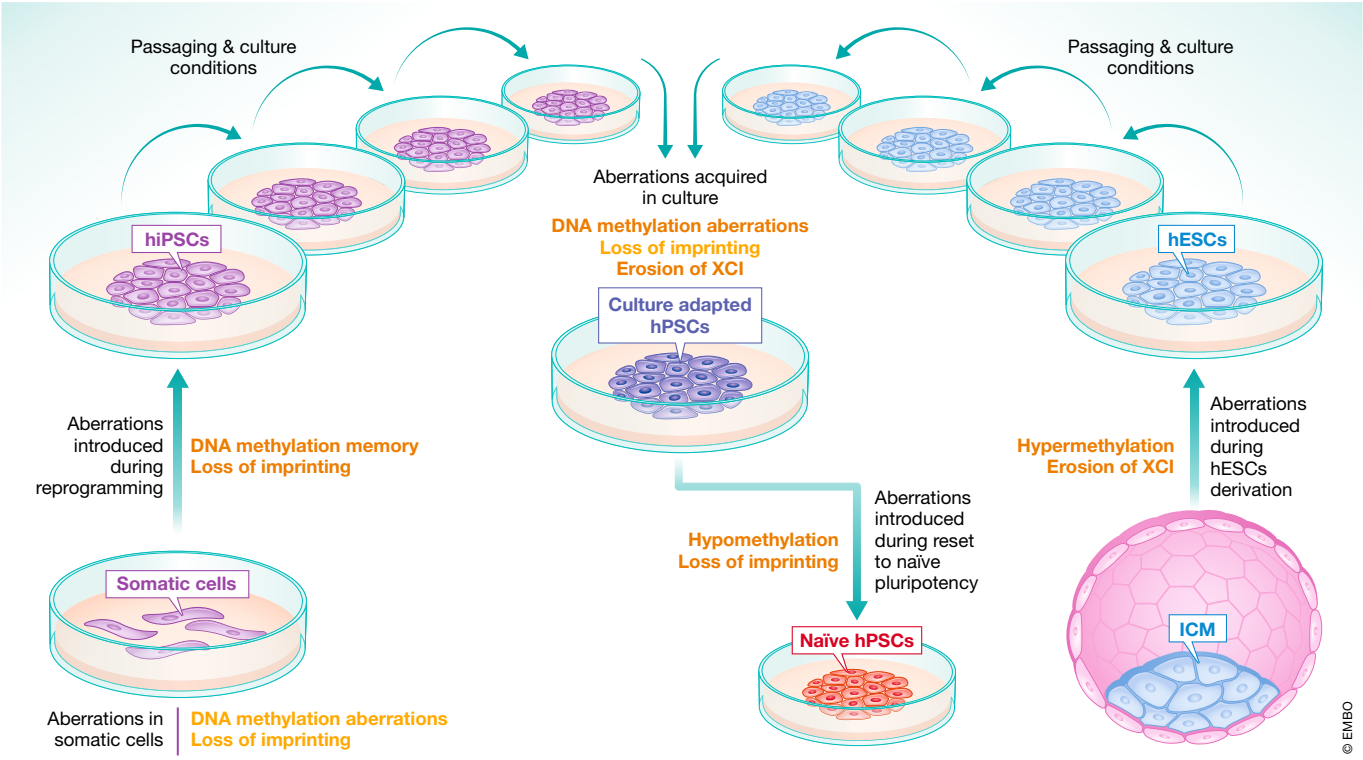

Figure 2. Origins of epigenetic aberrations in hESCs and hiPSCs.

5-methylcytosine (5-mC) aberrations accumulate following continuous growth in culture. Newly reprogrammed hiPSCs preserve somatic DNA methylation memory. Loss of imprinting (LOI) emerges mainly during reprogramming and transition to naïve pluripotency, but can also spread in culture of hPSCs and somatic cells. Erosion of X chromosome inactivation (XCI) in hESCs appears during their derivation and becomes widespread in very early passages. hiPSCs maintain XCI in early passages, but erosion can occur over time. Dark color represents *in vitro* processes that are more frequently associated with the epigenetic aberration.

2011; Nishino *et al*, 2011; Koyanagi-Aoi *et al*, 2013; Planello *et al*, 2014; Nishino & Umezawa, 2016; Tesarova *et al*, 2016). It was shown that employing different combinations of reprogramming factors result in distinct patterns of 5-mC aberrations. Reprogramming with Yamanaka factors (OCT4, SOX2, KLF4, and cMYC) is mostly associated with increased methylation in specific regions, while Thomson factors (OCT4, SOX2, NANOG, and LIN28) induce reduced methylation in different locations (Planello *et al*, 2014). This suggests that induction of such abnormalities is possibly due to the massive epigenetic perturbation which is provoked in this process (Liang & Zhang, 2013; Fig 2). Importantly, some of these methylation aberrations are also implicated in different tumors (Ohm *et al*, 2010) and are transmitted throughout differentiation (Lister *et al*, 2011; Ruiz *et al*, 2012). Nevertheless, while somatic memory and aberrations in DNA methylation exist in early-passage iPSCs, some studies found that many of them were diminished over time, at which point iPSCs become highly similar to ESCs in their methylation pattern (Nishino *et al*, 2011; Nishino & Umezawa, 2016; Tesarova *et al*, 2016), whereas others demonstrated a preservation of epigenetic memory over time (Kim *et al*, 2011). Even though reprogramming is often considered the cause for acquiring methylation aberrations, it was shown that it can also enable the reversal of 5-mC alterations in some tumor suppressors and cancer-related genes, which were abnormally methylated and silenced in somatic cells (Ron-Bigger *et al*, 2010). Overall, various studies illustrate the dynamic regulation of DNA methylation in iPSCs, yet these variations are mostly stabilized during prolonged growth in culture. However, while reprogramming-related methylation aberrations are resolved at high passages, growth-related changes could also be selected at the same time, raising a conflict regarding the recommended culture practice for human iPSCs.

Analysis of DNA methylation

DNA methylation can be inspected at various resolutions for unraveling the distribution, specificity, stability, and effects of this modification. In the past, treating extracted DNA with methylation-sensitive enzymes (Cedar *et al*, 1979) followed by PCR amplification facilitated the regional analysis of 5-mC changes at specific genomic locations. In order to extend this resolution and obtain single nucleotide information, additional methods were developed based on bisulfite treatment, which specifically converts unmethylated cytosine to uracil, allowing the distinction between methylated and unmethylated CpGs. Bisulfite sequencing PCR or pyrosequencing implements this distinction to obtain single CpG data for similar loci-specific analysis (Frommer *et al*, 1992; Tost & Gut, 2007; Table 1). However, these techniques require prior knowledge of putative genes which are susceptible to aberrations. Thus, an unbiased screen of DNA methylation aberrations calls for a genome-wide evaluation of 5-mC at single nucleotide resolution. Formerly, this was mostly established by employing DNA methylation arrays, in which multiple probes enable

the analysis of methylation at various genomic regions (Gitan *et al*, 2002; Weber *et al*, 2005), or by reduced representation bisulfite sequencing (RRBS), which combines bisulfite treatment with specific restriction enzymes to quantify methylation at regions with high CpG content (Meissner *et al*, 2005). Lately, the significant cost reduction of whole-genome sequencing is driving more laboratories to apply analysis of DNA methylation across the entire genome, by executing whole-genome bisulfite sequencing (WGBS; Lister *et al*, 2009; Table 1). More recently, WGBS was also performed in single cells (Smallwood *et al*, 2014), enhancing the sensitivity of such analyses. A newly established DNA methylation reporter offers investigation of 5-mC in single live cells, indicating dynamic methylation changes at specific loci (Stelzer *et al*, 2015).

While the abovementioned methods are useful for analyzing 5-mC, it does not readily indicate transcriptional alterations. Therefore, combining these methods with RNA quantification, as recently performed in parallel single-cell sequencing (Angermueller *et al*, 2016), is highly valuable for associating changes in methylation and gene expression, to ultimately achieve a comprehensive picture of DNA methylation aberrations in hPSCs.

Parental imprinting

Parental imprinting is a phenomenon that evokes monoallelic epigenetic silencing of certain genes in a parent-of-origin mode by incorporating 5-mC to discriminate between the maternal and paternal alleles in mammalian cells. This is enabled by the establishment of differentially methylated regions (DMRs) at specific loci of the oocyte and sperm genomes, which are subsequently maintained in the zygote and throughout development. These DMRs directly regulate the expression of nearby genes, leading to monoallelic silencing of ~ 100 imprinted genes (Bartolomei & Ferguson-Smith, 2011). Elegant nuclear transfer experiments that enforced activation of oocytes containing same-sex genomes demonstrated that imprinting poses the main barrier for uniparental reproduction in mammals (McGrath & Solter, 1984; Surani *et al*, 1984). Only extensive artificial editing and removal of several imprinted regions could overcome this impediment and successfully generate all-maternal (parthenogenetic), and recently also all-paternal (androgenetic) mice (Kono *et al*, 2004; Kawahara *et al*, 2007; Li *et al*, 2018). In humans, spontaneous parthenogenetic and androgenetic development may commence, but result in tumors. Moreover, loss of imprinting (LOI) at specific loci leads to developmental disorders such as Prader–Willi syndrome and Beckwith–Wiedemann syndrome. Nevertheless, imprinting is considered highly stable throughout life and across tissues, with only a few examples of tissue-specific imprinting mostly observed in the placenta (Frost & Moore, 2010). However, various studies identified considerable incidence of LOI in hPSCs, resulting in the expression of imprinted genes from both alleles instead of one, along with DNA methylation changes at imprinting DMRs (Rugg-Gunn *et al*, 2005; International Stem Cell Initiative *et al*, 2007; Pick *et al*, 2009; Nazor *et al*, 2012; Ma *et al*, 2014; Johannesson *et al*, 2014; Bar *et al*, 2017; Fig 1). Many imprinted genes reside in clusters and are conversely regulated by the same germline DMR. Thus, alteration in a single region can drive both biallelic expression and silencing of multiple genes (Bartolomei & Ferguson-Smith, 2011). Notably, imprinting aberrations persist following differentiation toward various cell types, thereby aggravating the consequences of LOI on hPSCs growth and integrity.

Table 1. Methodologies for detecting epigenetic aberrations.

Method	Resolution	DNA methylation aberrations	Loss of imprinting	XCI erosion
High-throughput RNA sequencing	Single nucleotide; Genome-wide; Optional single cells	Gene silencing/activation	Allelic expression; Quantified expression levels of imprinted loci	*XIST* expression; Allelic expression (X chromosome); Quantified expression levels of X-linked genes
WGBS	Single nucleotide; Genome-wide; Optional single cells	DNA methylation changes	DNA methylation levels at DMRs	DNA methylation levels across X chromosome
RRBS/Methylation arrays	Single nucleotide; Multiple genomic regions	DNA methylation changes	DNA methylation levels at DMRs	DNA methylation levels across X chromosome
SNP arrays (DNA and RNA)	Single nucleotide; Multiple genes	N/A	Allelic expression	Allelic expression
Sanger sequencing (for identifying expressed SNPs)	Single nucleotide; Gene-specific	N/A	Allelic expression	Allelic expression
Bisulfite sequencing PCR/Pyrosequencing	Single nucleotide; Gene-specific	DNA methylation levels	DNA methylation levels at a DMR	N/A
Methylation-sensitive enzymatic restriction and PCR	Regional; Gene-specific	DNA methylation levels	DNA methylation levels at a DMR	N/A
RNA-FISH	Regional; Gene-specific; Single cells	N/A	Allelic expression	*XIST* coating; Cot-1 exclusion; Allelic expression
Immunofluorescence	Protein-specific; Single cells	N/A	N/A	H3K27me3 puncta; RNA-Pol II exclusion

FISH, fluorescent *in situ* hybridization; N/A, not applicable; PCR, polymerase chain reaction; RRBS, reduced representation bisulfite sequencing; SNP, single nucleotide polymorphism; WGBS, whole-genome bisulfite sequencing.
Color shades represent the strength of the method in detecting specific epigenetic aberrations (dark orange—high strength).

The sources for loss of parental imprinting

hPSCs acquire distinct DNA methylation signatures as compared to their cell type of origin (as detailed above). These epigenetic dynamics are a potential cause for the multiple reports on LOI in hPSCs. While studies examining the status of imprinted genes in ESCs concluded that they are for the most part stable and have relatively rare instances of LOI (International Stem Cell Initiative *et al*, 2007; Rugg-Gunn *et al*, 2007), inspection of iPSCs or SCNT-PSCs identified higher degrees of imprinting aberrations in these cells (Pick *et al*, 2009; Johannesson *et al*, 2014; Ma *et al*, 2014). Recently, a large-scale analysis of LOI in hundreds of hPSC samples was performed. The results corroborated significantly higher levels of LOI in iPSCs compared with ESCs (Bar *et al*, 2017). This analysis also revealed that some imprinted genes are already expressed biallelically in a proportion of parental fibroblast cells used for reprogramming, which is maintained and even expanded in some of the hiPSC lines, since they are derived from single-cell clones. Overall, these results suggest that imprinting is lost primarily during the reprogramming process, but also upon culturing fibroblasts and hPSCs (Fig 2). Nevertheless, while DNA methylation aberrations in most genes were shown to be relieved in iPSCs upon culture adaptation (Nishino *et al*, 2011; Nishino & Umezawa, 2016; Tesarova *et al*, 2016), the differential methylation at imprinted regions cannot be restored since restoration requires the distinction between maternal and paternal alleles, which is feasible only in the gametes. Furthermore, recent studies in mouse and human PSCs suggest that globally, DNA methylation in these cells is highly dynamic, cycling between *de novo* methylation and its erasure (Shipony *et al*, 2014; Rulands *et al*, 2018). However, this turnover is not observed in imprinted regions (Shipony *et al*, 2014), suggesting a special regulation for maintaining imprinting in PSCs.

Collectively, these findings indicate that DMRs are more susceptible to aberrations during somatic cell reprogramming toward pluripotency, while being more tightly preserved in standard culture. In naive hPSCs, DNA methylation is substantially reduced, reaching similarly low levels as in pre-implantation development. However, contrary to the tight conservation of imprinting in the early embryo, methylation at most imprinted regions is lost in naive hPSCs of all current protocols (Pastor *et al*, 2016; Theunissen *et al*, 2016). Recent investigations also consistently discovered global erasure of DMRs in mouse naive PSCs following prolonged culture in 2i medium (Choi *et al*, 2017), propounding that further effort is required to improve these culture conditions as to prevent LOI.

In addition to differences in LOI rates between various hPSC types, the tendency of different imprinted genes to exhibit biallelic expression also poses an intriguing comparison. Several lines of evidence establish that while some genes are mostly resistant to imprinting aberrations (*SNRPN*, *PEG3*), other genes feature frequent LOI across many hPSC lines (*H19*, *IGF2*, *MEG3*, *ZDBF2*; Kim *et al*, 2007; Nazor *et al*, 2012). The main difference between genes with distinct LOI frequencies was linked to the parent-of-origin methylation regulating their monoallelic expression: Imprinted genes governed by a paternally methylated DMR are much more prone to LOI as compared to those controlled by a maternally methylated DMR (Rugg-Gunn *et al*, 2007; Bar *et al*, 2017). Particularly, this difference is prominent in iPSCs, signifying that imprinted paternally methylated regions are more sensitive to aberrations during reprogramming. This implies that maternal and paternal alleles might incorporate different mechanisms for protecting imprinted regions from demethylation. A supportive indication for this suggestion is observed in the zygote, where maternal and paternal pronuclei are both extensively demethylated after fertilization, but at distinct rates, and thus they may recruit different pathways to regulate this process and maintain parental imprinting. Yet, another stimulating possibility for resolving the varying sensitivity toward aberrations of different imprinted genes was suggested to involve correlation with the exact developmental timing of gene activation, whereby prominent transcription during early pre-implantation stages is associated with increased safeguarding of imprinting in hPSCs (Rugg-Gunn *et al*, 2007). Interrogation of the exact expression time point of additional imprinted genes, which were implicated in more recent studies of LOI, is required to better assess this hypothesis.

While LOI which emerges during reprogramming could take over the culture by means of clonal expansion, its spontaneous appearance during hPSCs growth is thought to occur rarely, only in a small subset of cells. Therefore, in order for the biallelic expression to spread, it should probably offer these cells a selective advantage of some sort. Such beneficial effect may include faster proliferation, reduced apoptosis, decreased differentiation, and increased colony formation. Biallelic activation of an imprinted gene is expected to cause a twofold increase in its expression, but while some genes follow this simple linear model, others are transcribed at higher (*RTL1*, *IGF2*) or lower (*PEG10*, *SGCE*, *NDN*) levels than anticipated, indicative of a more complex regulatory network (Bar *et al*, 2017). Correspondingly, upregulations of several imprinted genes, especially those with high rates of LOI in hESCs (e.g., *IGF2*, *PEG10*, *DLK1*), were implicated in various malignancies (Huang *et al*, 2007; Xu *et al*, 2012; Li *et al*, 2014; Brouwer-Visser & Huang, 2015; Xie *et al*, 2018). Moreover, LOI is known to be highly frequent in many types of cancers, though accumulative evidence suggests it appears early and originates in the stem cell niche of the tissue which sourced the tumor. In these stem cells, LOI presumably contributes to an increase in the stem cell pool and thus elevates the chances for a genetic mutation driving the cancerous transformation of these cells (Jelinic & Shaw, 2007; Leick *et al*, 2012). Furthermore, secretion of IGF2, which is encoded by an imprinted gene, was found to be important for hPSCs self-renewal (Bendall *et al*, 2007) as well as for proliferation of several adult stem cells (Barroca *et al*, 2017; Youssef *et al*, 2017). These results suggest a special role for imprinted genes in regulating stem cell properties, therefore supporting the speculation that LOI might promote hPSC growth and survival in culture.

Taken together, various analyses suggest that imprinting aberrations emerge primarily during somatic cell reprogramming at paternally imprinted regions, but also during growth in culture, in which LOI is selected due to the upregulation of growth-promoting genes or the silencing of growth-restricting genes. These observations strengthen the need for further research to identify potential regulators of imprinting in early human development, which will also assist in improving the conditions for reprogramming and culturing hPSCs to minimize imprinting aberrations.

Determining the status of parental imprinting

Since imprinted genes are expressed monoallelically, LOI may result in either their abnormal silencing or biallelic expression, mostly by methylation changes at imprinted regions. Thus, detecting imprinting aberrations is feasible by analyzing RNA sequence, expression

levels, and/or DNA methylation by various methods that differ in resolution, ranging from high-throughput (e.g., high-throughput RNA sequencing, WGBS), medium-throughput (e.g., expression SNP and DNA methylation arrays), or low-throughput (e.g., Sanger sequencing, RNA fluorescent *in situ* hybridization, methylation-sensitive enzymes), facilitating LOI discovery in either multiple or single imprinted genes (Table 1).

To infer LOI, RNA sequencing could be interrogated for identifying biallelic expression of imprinted genes. This involves the detection of single nucleotide polymorphisms (SNPs) in the RNA transcripts, indicative of expression from both maternal and paternal alleles. Still, this method is dependent on an evident transcription of the gene in the analyzed cells, as well as on the existence of a polymorphic site within the expressed region. Hence, ruling out LOI is possible only if such a SNP was verified at the DNA level. Furthermore, expressed SNPs should be classified cautiously, following the consideration of possible confounding variables such as sequencing noise and coverage. In addition, identifying biallelic silencing of imprinted genes due to LOI by RNA analyses is not straightforward, this is because various changes in transient regulatory factors could cause such silencing, but most of them do not affect the imprinting of a gene.

Alternatively, analyzing DNA methylation at imprinted regions is feasible for ascertaining LOI even when it drives gene repression and does not require genetic polymorphisms (Nazor *et al*, 2012). However, in some cases, the imprinted monoallelic expression of a gene might be lost due to improper H3K27me3 acquisition, without apparent modifications to DNA methylation (Zhang *et al*, 2011). Furthermore, the degree of modification in DNA methylation required to drive LOI is mostly unknown. Also, when only a subpopulation of cells within the culture harbor imprinting aberrations, its detection by RNA analysis might be more sensitive than probing bulk methylation.

X chromosome inactivation

In all mammals, dosage compensation of X-linked genes between males and females is achieved by X chromosome inactivation (XCI). This epigenetic process results in the silencing of most genes on one randomly chosen X chromosome of female somatic cells, triggered by the transcription and consecutive coating of the long non-coding RNA *XIST* on the inactive X (Xi). Nevertheless, recent data indicate significant differences in the exact mechanism, timing, and regulation of XCI between mammalian species, specifically human and mouse, both *in vivo* and *in vitro* (Payer & Lee, 2014; Sahakyan *et al*, 2017b). ESCs and iPSCs generated from female mice reflect a pre-XCI state as in mouse blastocysts, in which *Xist* is not expressed and both X chromosomes are active (XaXa). However, recent analyses of early human embryos indicate that *XIST* is expressed in human ICM cells along with apparent dosage compensation (Petropoulos *et al*, 2016). Nevertheless, there is still a debate as to whether in these cells, X-linked genes are expressed mostly monoallelically, representing initiation of XCI, or biallelically, reflecting the dampening of expression by an unknown mechanism (Petropoulos *et al*, 2016; Moreira de Mello *et al*, 2017).

Multiple studies revealed that hPSCs exhibit different states of XCI, which vary across cell lines (Hoffman *et al*, 2005; International Stem Cell Initiative *et al*, 2007; Shen *et al*, 2008; Silva *et al*, 2008; Dvash *et al*, 2010; Bruck & Benvenisty, 2011; Lessing *et al*, 2013; Patel *et al*, 2017; Sahakyan *et al*, 2017b). In some hPSCs, *XIST* is expressed and one X chromosome is completely inactive (XaXi). Yet other hPSCs, in which *XIST* is silenced, could be further differentiated by those having either two active X chromosomes (XaXa, as in mice) or cells experiencing erosion of XCI (XaXe; Fig 1). XCI erosion is highly frequent and leads to loss of H3K27me3 and DNA methylation at the Xi, along with biallelic expression and upregulation of several X-linked genes (Silva *et al*, 2008; Fig 1). This is considered an abnormal epigenetic alteration, since it differs from any known *in vivo* representation of the X chromosome, in both undifferentiated and somatic cells. Critically, various studies showed that hPSCs do not change their XCI state upon differentiation. Thus, *in vitro* derived somatic cells may express abnormally elevated levels of X-linked genes (Patel *et al*, 2017; Sahakyan *et al*, 2017a). Notably, hPSCs that silence one X chromosome either partially (XaXe) or completely (XaXi) do not mirror random XCI as exhibited in normal development, but demonstrate mostly XCI skewing, in which the same X chromosome is silenced in all cells of a given hPSC line (Geens & Chuva De Sousa Lopes, 2017; Sahakyan *et al*, 2017a).

The basis for variability in X chromosome inactivation

In humans, *XIST* is expressed in most stages of female development, including during pre-implantation development (morula and blastocyst) and in practically all somatic cells. In hPSCs, *XIST* loss is associated mostly with erosion of XCI, which seems to be widespread among various hPSC lines (Vallot *et al*, 2015; Geens & Chuva De Sousa Lopes, 2017; Sahakyan *et al*, 2017b). Close inspection of the isolation of ESCs from human blastocysts revealed that silencing the expression of *XIST* in these cells occurs rapidly at very early passages (Geens *et al*, 2016; Patel *et al*, 2017). In contrast, reprogramming somatic cells to iPSCs does not induce this rapid suppression of *XIST* expression and most iPSCs are reported to maintain X inactivation as in their cells of origin (Tchieu *et al*, 2010; Pomp *et al*, 2011). However, some iPSCs do tend to downregulate *XIST* in culture (Tchieu *et al*, 2010; Pomp *et al*, 2011; Anguera *et al*, 2012; Mekhoubad *et al*, 2012; Fig 2).

Overall, there seems to be a striking predilection toward abolishing *XIST* transcription in hPSCs, yet currently the driving forces for this phenomenon remain elusive. Several hypotheses might explain the abundance of XCI erosion in hPSCs. X chromosome duplications have been observed as a relatively frequent chromosomal aberration in hPSCs, indicative of positive selective advantage toward an increased dosage of X-linked genes (Baker *et al*, 2007). However, the upregulation of these genes, as a result of chromosomal duplication, is not as straightforward as in autosomes, in that extra copies of the X chromosome are known to be subjected to XCI *in vivo*. Thus, the seemingly beneficial genes belong either to those which escape XCI or to the genes which are affected by erosion. In mouse ESCs, two active X chromosomes are associated with delayed differentiation, reduced methylation, and upregulation of pluripotency factors (Schulz *et al*, 2014). Furthermore, various genes which are overexpressed following erosion were shown to be upregulated in cancer, supporting their putative role in enhancing self-renewal (Anguera *et al*, 2012). Altogether, these observations suggest that erosion of XCI may lead to increased levels of some X-linked transcripts which contribute to the growth and survival of hPSCs. While overexpression of advantageous genes might contribute to the loss of *XIST* in culture, it does not readily explain the fast kinetics of its near complete shut-off during hESC derivation.

Alternatively, the selection against a *XIST*-coated X chromosome might be affected by its delayed replication (Willard & Latt, 1976; Gómez & Brockdorff, 2004). This is implicated in many types of female cancers, in which the inactive X chromosome is absent, commonly accompanied by duplication of the active X (Kawakami *et al*, 2004). Yet, female hPSCs are rarely deprived of one X chromosome, possibly accounting for important dosage-dependent expression of some genes which escape X inactivation. Thus, substantial delay in replication of an entire chromosome may better explain the swift erosion in hESCs.

Another possible explanation proposes that the expression of *XIST* might be negatively regulated by pluripotency-related genes. Indeed, in mice, *Xist* was reported to be suppressed by multiple pluripotency factors (Augui *et al*, 2011) and its activation correlated with low levels of the pluripotency gene *Nanog* (Sousa *et al*, 2018). However, unlike in humans, *Xist* is completely absent from mouse blastocysts and PSCs. Therefore, its evident downregulation by the pluripotency network in mice is probably not exactly recapitulated in humans *in vivo*. Nevertheless, *XIST* silencing might be facilitated following an artificially prolonged expression of such pluripotency regulators (as in hPSCs *in vitro*). Still, the link between pluripotency and *XIST* activation in humans is largely unknown and further investigations are required to evaluate it.

Notably, the exact molecular mechanism directing the repression of *XIST* is also for the most part obscure. In mouse somatic cells, *Xist* repression was shown to be dependent on DNA methylation at its promoter (Norris *et al*, 1994). Male mice deficient in DNA methyltransferases (DNMTs) exhibit an aberrant expression of *Xist* from their single X chromosome (Beard *et al*, 1995; Panning & Jaenisch, 1996). Accordingly, treating somatic human/mice hybrid cells or HCT116 colon cancer cell line with the demethylating agent 5-aza-dc led to hypomethylation at the *XIST* promoter, along with its subsequent upregulation from the active human X chromosome (Tinker & Brown, 1998; Vasques *et al*, 2005), indicating its dependence on DNA methylation also in human somatic cells. However, undifferentiated male mouse ESCs did not elevate *Xist* following similar knockout of DNMTs (Beard *et al*, 1995); hence in these cells, demethylation is insufficient for driving *Xist* upregulation, probably due to its suppression by the abovementioned pluripotency network. Nonetheless, XCI erosion and *XIST* silencing in hPSCs were strongly correlated with elevated methylation at the *XIST* promoter (Shen *et al*, 2008; Xie *et al*, 2016). Moreover, 5-aza-dc treatment in eroded H7 hESCs induced the re-expression of *XIST*, but was insufficient for its proper coating of the X chromosome (Hoffman *et al*, 2005). This suggests that DNA methylation is indeed involved in *XIST* silencing during XCI erosion in hPSCs, but also points to additional factors which are required for its correct function.

Additionally, recent studies on naive female hPSCs, which sustain genome-wide demethylation, illustrated upregulation of *XIST*. However, *XIST* formed a diffuse cloud on the X chromosomes, different than the tight cloud which is observed in somatic cells. This pattern of *XIST* coating is followed by biallelic expression of X-linked genes in these cells, indicative of two active chromosomes (XaXa; Sahakyan *et al*, 2017a; Vallot *et al*, 2017). These features better reflect the X chromosome properties observed in human blastocysts. Furthermore, during the course of differentiation, these naive hPSCs transiently silence *XIST* (as they transit through a primed state), but then later re-express it, driving XCI in their differentiated derivatives. Importantly, this was demonstrated also for eroded hPSC lines that normally are not able to upregulate *XIST* following differentiation. Nevertheless, naive hPSCs failed to promote random XCI in their differentiated cells, which instead exhibit skewed silencing of the same X chromosome that was eroded or inactive in the original hPSC line (Sahakyan *et al*, 2017a). This skewing indicates a strong epigenetic memory of Xi. Thus, although current protocols for generating naive hPSCs offer progress in normalizing XCI aberrations, further improvements are still required to completely resolve them.

Alternatively, growing hESCs in physiological oxygen levels (5%) was suggested as another culture modification to sustain hPSCs with two active X chromosome and to prevent XCI erosion (Lengner *et al*, 2010). Remarkably, these conditions also facilitate random XCI following differentiation. Yet, this requires hESCs to be derived under low oxygen concentration, or at least to be cultured as such at very low passages before initiation of XCI, as it cannot reactivate the inactive or eroded X chromosome in culture or during reprogramming (Lengner *et al*, 2010; Tchieu *et al*, 2010).

Differentiating between X chromosome inactivation states

The great variability in XCI among hPSCs can be reflected by different measurements, including distinct *XIST* expression levels, DNA methylation signatures, accumulation of repressive histone modifications, and inconsistent number of X-linked genes exhibiting monoallelic transcription and apparent dosage compensation. All of these features can be measured by various methods (Table 1), but the accurate determination of XCI state requires a combined analysis of several components. For example, the expression of *XIST* can be detected by RNA fluorescent *in situ* hybridization (RNA-FISH) or by RNA sequencing (RNA-Seq; Table 1), yet its absence cannot indicate whether a cell contains two active X chromosomes (XaXa) or one eroded X chromosome (XaXe; Silva *et al*, 2008). Since in XCI erosion certain chromosomal regions remain silenced (Vallot *et al*, 2015), the distinction between an active or eroded X chromosome requires a more profound analysis of X-linked genes to inspect their allele specificity and degree of expression. High-throughput RNA-Seq offers an attractive technique to capture several properties at once, as it enables both quantification and sequence interrogation of transcribed RNA. Thus, three different elements could be extracted from RNA-Seq data simultaneously, including *XIST* levels, global dosage compensation, and biallelic expression along the X chromosome. Nevertheless, visualization of *XIST* and other X-linked genes by RNA-FISH is also highly beneficial, since it facilitates interrogation of XCI in single cells. This is particularly critical if cultured cells exhibit random inactivation, which hinders the identification of monoallelic signal in bulk RNA-Seq. Additionally, this method provides means to assess the functionality of *XIST* RNA by inspecting its coating of the X chromosome. Accordingly, the appearance of *XIST* as a tight cloud is indicative of XCI initiation, while its dispersion is suggestive of impaired activity (Yue *et al*, 2014). Alternatively, the improvements and increased implementation of single-cell RNA-Seq (Lao *et al*, 2009) could also expedite the investigation of XCI in single cells (Deng *et al*, 2014).

To further obtain a general assessment of XCI in single cells, additional imaging methods can be implemented (Table 1). Immunostaining of repressive histone marks which accumulate on the inactive X such as H3K27me3 can also add information on the functionality of

XIST (Yue *et al*, 2014). An additional indication can be obtained by applying RNA-FISH for Cot-1 expression, which is based on the finding that repetitive DNA sequences are allocated to the nucleoplasm, from which they are transcribed in all active chromosomes, but are uniquely silenced in the Xi (Hall & Lawrence, 2010).

Additional epigenetic features

Epigenetic modifications are mostly associated with changes in gene expression, which were shown to differ between hPSCs grown in different microenvironments (Newman & Cooper, 2010), as well as between hESCs and hiPSCs (Chin *et al*, 2009, 2010) and between primed and naïve hPSCs (Messmer *et al*, 2019). However, quantifying the transcriptional levels of genes in the pluripotent state does not directly reveal the underlying mechanism of variability, nor its level of stability and inheritance. In this review, we discuss gene expression alterations in the context of specific epigenetic mechanisms which are known to drive them.

Posttranslational modifications of histone tails are important epigenetic regulators, controlling activation and repression of gene expression. Specifically, PSCs harbor a unique bivalent chromatin, in which both repressing and active histone modifications are present in the same genomic region, mostly associated with promoters of developmental genes. This results in poised genes, which are silenced in the pluripotent state but are proned toward rapid activation upon differentiation (Vastenhouw & Schier, 2012). Specifically, repressing developmental genes by trimethylation of H3 on Lysine 27 and Lysine 9 (H3K27me3 and H3K9me3) have important roles in hPSCs (Lee *et al*, 2006; Pan *et al*, 2007; Zhao *et al*, 2007). While H3K27me3 was shown to be consistently similar between hESCs and hiPSCs, distinct H3K9me3 regions are evident in hiPSCs, due to both somatic epigenetic memory and reprogramming-induced aberrations (Chin *et al*, 2009; Guenther *et al*, 2010). Furthermore, differential H3K9me3 signatures resulted in greater gene expression differences between these cell types (Hawkins *et al*, 2010). Nevertheless, analyses of histone modifications in additional hPSC lines are required in order to conclude the degree and mechanism of their heterogeneity.

Consequences of epigenetic aberrations for hPSCs utilities

Studying human development

Research on human development is extremely limited, especially during early post-implantation stages, as they are mostly inaccessible for detailed analysis. Thus, many studies are performed in mice as a major model organism for mammalian development. However, there are significant differences between mice and humans during the course of implantation (Dvash & Benvenisty, 2004; Rossant, 2015). Consequently, hPSCs are highly valuable in investigating processes which occur during early human embryogenesis and exploring signaling pathways for lineage specification. Nevertheless, some of the epigenetic aberrations discussed above might impact the utility of hPSCs for this purpose and require special attention under certain conditions, which are detailed herein (Fig 3).

DNA methylation variations in promoters of genes that are important for development may affect the differentiation propensity of hPSCs toward specific lineages. Consequently, quantifying DNA methylation and gene expression can predict the ability of a given hPSC line to differentiate toward a specific cell type (Bock *et al*, 2011). Additionally, aberrant hypomethylation followed by activation of endogenous retroviruses in hiPSCs leads to impaired differentiation (Koyanagi-Aoi *et al*, 2013). Furthermore, somatic epigenetic memory, which is retained in iPSCs, was also shown to constitute a differentiation bias, usually toward the lineage of the source cell which was used for reprogramming (Kim *et al*, 2011). For example, iPSCs derived from fetal brain preserved DNA methylation patterns characteristic of brain tissue, and subsequently exhibited a higher inclination toward producing neural cells (Roost *et al*, 2017). In another study, human beta cells were used for reprogramming, generating iPSCs which presented a higher competence for differentiating into insulin-producing cells, either *in vivo* or *in vitro* (Bar-Nur *et al*, 2011). Nonetheless, the efficiency of iPSCs toward hematopoietic commitment was not associated with cell type of origin, but further hematopoietic maturation was found to be altered by methylation aberrations which are acquired during reprogramming to iPSCs (Nishizawa *et al*, 2016). Therefore, in studies of human development which involve differentiating hPSCs, such propensity toward specific derivatives could distort the genuine results and lead to misinterpretation (Fig 3). Specifically, research which might be affected by such prejudice includes establishing a new differentiation protocol, determining the effect of signaling molecules and transcription factors on lineage specification, and investigating the developmental consequences of mutations *in vitro*. Therefore, using late-passage hiPSCs (in which the epigenetic memory is mostly ameliorated) in such studies might prevent potential bias in such analyses.

LOI is directly linked with abnormal embryonic development, as demonstrated by the inability of parthenogenetic and androgenetic conceptuses to survive past early stages of gestation, by which they can be transformed into tumors in human (Linder *et al*, 1975; Kajii & Ohama, 1977; Fig 3). These tumors are comprised of distinct tissues, which in the case of parthenogenetic ovarian teratoma includes disorderly embryonic structures of the three germ layers (Stamp & McConnell, 1983), while androgenetic hydatidiform mole constitutes cells of extraembryonic origin (Candelier, 2016). Further studies in mouse chimeras confirmed that lacking either the maternal or paternal genomes contributes to the formation of discrete cell types, recapitulating the bias toward embryonic and trophoblast tissues exhibited in bimaternal and bipaternal embryos, respectively (Thomson & Solter, 1988). Moreover, close inspection of the chimera brains identified alternate contributions of androgenetic and parthenogenetic cells into distinct brain regions, implying an even more complex role for imprinted genes in cellular specification (Davies *et al*, 2005). Differentiating parthenogenetic hPSCs also revealed impaired specification of liver and muscle tissues, as well as significant deficiency of placenta-specific genes compared to wild-type hPSCs following spontaneous and directed differentiations (Stelzer *et al*, 2011). In addition to genome-wide LOI, as exemplified by uniparental cells, LOI in specific imprinted regions was also shown to influence the bias toward specific cell types (Fig 3). For instance, overexpression of necdin, the protein product of the paternally expressed gene *Ndn*, enhances the differentiation of forebrain GABAergic neurons in mouse brain culture (Kuwajima *et al*, 2006). Correspondingly, Prader–Willi syndrome patients, in which *NDN* is not expressed, exhibit irregular GABA signaling that is considered to have a major role in the phenotype of this neurodevelopmental disorder (Dimitropoulos *et al*, 2000). In addition, this gene has

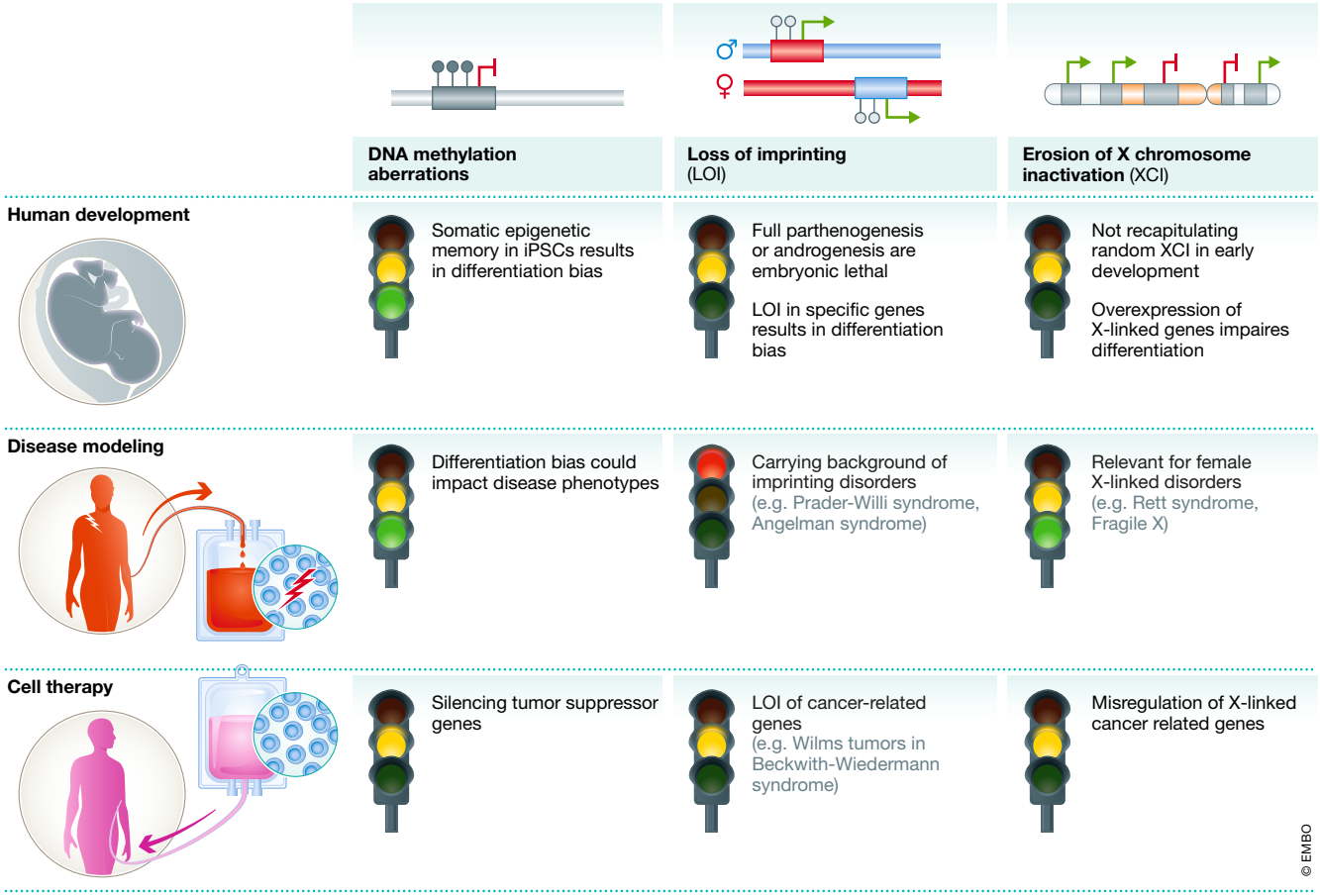

Figure 3. Consequences of epigenetic aberrations on hPSCs applications.

A summary of the evident consequences of epigenetic aberrations for utilizing hPSCs in studying human development, disease modeling, and cell therapy. Traffic light colors represent the severity of such influences (red being the most severe). LOI, loss of imprinting; XCI, X chromosome inactivation.

important functions in axonal growth (Lee *et al*, 2005), demonstrating yet another possible developmental implication for losing its imprinting. In patients with Angelman syndrome, silencing the expression of the maternally expressed gene *UBE3A* results in brain structural aberrations, such as cortical atrophy and Purkinje cell loss (Davies *et al*, 2005). Collectively, these results substantiate the importance of using hPSCs in which parental imprinting is intact, for studying various aspects of human development.

XCI initiation occurs during early human embryogenesis, between pre-implantation and post-implantation. Recent research on human blastocysts suggested X chromosome dampening in human pre-implantation (Petropoulos *et al*, 2016). Potentially, hPSCs could have been an ideal model to study XCI, similar to other developmental processes. However, the clear divergence in their XCI properties when compared to human ICM cells (Geens & Chuva De Sousa Lopes, 2017), which is followed by their difficulty to experience random XCI *in vitro*, prohibits their use for interrogating this process. Moreover, the erosion of XCI results in reduced differentiation efficiency *in vitro* and *in vivo* (Silva *et al*, 2008; Anguera *et al*, 2012; Patel *et al*, 2017; Fig 3). Correspondingly, this overexpression also generates an impaired dosage compensation between the sexes, which could lead to erroneous differences between male and female hPSCs when studying human development. While we do not suggest that female hPSCs should not be used to study human development, their improper XCI signal should be considered upon analyzing the results.

Disease modeling

Modeling human diseases is critical for understanding their underlying molecular mechanisms and for enabling screening of potential therapeutic drugs. While model organisms are valuable in studying many human disorders and malignancy, there are also various examples in which they do not manifest the human phenotype, or in which the associated genes and molecular pathways are not recapitulated. The increase in sophisticated techniques to investigate and manipulate cells *in vitro* has promoted the linking of genetic and epigenetic alterations that trigger diseases with their effect on cells and tissues in culture. hPSCs are highly beneficial for this purpose, as they combine the ability to be propagated indefinitely, while at the same time they do not require immortalization and can be maintained with mostly an intact genome (Avior *et al*, 2016). The ability to reprogram somatic cells from patients of a certain

disease and generate autologous hiPSCs, along with recent advancement in establishing hPSCs-derived organoids (Lancaster & Knoblich, 2014), extends their feasibility for studying complex phenotypes, which are related to the impact of human disorders on an entire tissue or organ. For these reasons, hPSCs are widely implemented in studying human syndromes and screening for possible drugs (Avior *et al*, 2016); however, this might be affected by different aberrations, including those of epigenetic source, which should thus be premeditated as discussed below.

DNA methylation aberrations in hPSCs were not shown to have a direct influence on human disease modeling in the pluripotent state. Nevertheless, lineage bias by aberrant methylation (as detailed above for studying human development) could be relevant if cellular differentiation is applied to assess cell-type-specific phenotypes related to the investigated disease (Fig 3).

Imprinting disorders are caused by misregulation of various imprinted regions, as a result of either uniparental chromosomal (or sub-chromosomal) disomy, genetic deletions, point mutations, or DNA methylation aberrations (Ishida & Moore, 2013). Most of these syndromes are characterized by LOI of multiple genes in the same locus, while the contribution of individual genes to the underlying phenotype is not always clear. For example, Prader–Willi syndrome involves a large cluster of paternally expressed genes, which are abnormally silenced in these patients and lead to complex neurodevelopmental phenotypes including characteristic facial features, chronic over-eating (hyperphagia) and obesity, short stature, and behavioral problems (Cassidy *et al*, 2012). Still, it is not yet known which specific gene/s drive the outcome of this disorder (Cheon, 2016). Another well-studied imprinting disorder is Beckwith–Wiedemann syndrome, which is characterized by variable features including macrosomia (significant overgrowth), macroglossia (enlarged tongue), and embryonal tumors (such as Wilm's tumor, neuroblastoma, hepatoblastoma; Brioude *et al*, 2018). Notably, more than 50% of patients diagnosed with this syndrome present either loss or gain of DNA methylation in DMRs regulating the imprinted genes KCNQ1/CDKN1C or H19/IGF2, respectively (Elhamamsy, 2017). Markedly, the same genes are also those classified as being highly aberrant in hPSCs. Thus, LOI in hPSCs could mirror the molecular features which are apparent in such imprinting diseases. Consequently, utilizing hPSCs as a model for studying human disorders could be seriously impaired if these cells are carriers of a different syndrome in their background, because phenotypes caused by the modeled or the imprinted diseases are indistinguishable. Thus, LOI should be carefully assessed in modeling diseases, since it might disrupt the desired discoveries. In addition, as more thoroughly explained in the next section, LOI seems to have an active role in cancer by promoting stem cell features and self-renewal. These influences might also override the phenotypes of the studied disease or alter the response toward potential drugs, therefore substantiating the need to implement LOI-free hPSCs for this purpose.

The unavailability of human brain tissues substantiates the need for a comprehensive model system for studying neurodevelopmental diseases such as those classified as autism spectrum disorders (ASD). hiPSCs are emerging as a great potential to study such disorders since they could be generated from ASD patients, thus reflecting their genetic and molecular landscape, along with the ability of these cells to differentiate to functional neurons or brain organoids *in vitro* (Prilutsky *et al*, 2014). Whereas ASDs are globally more frequent in males (Loomes *et al*, 2017), Rett syndrome (RTT) is a form of ASD apparent almost exclusively in females, which is caused by heterozygous mutations in the X-linked gene *MECP2*. Following XCI, about half of the cells express only the wild-type *MECP2*, while the other half express only the mutated form, generating a mosaic pattern of MECP2 function in RTT somatic cells. As detailed above, most hiPSCs do not reactivate the silenced Xi following reprogramming, thereby generating skewed clones in which all the cells express either the normal or mutated allele. This reinforces the need to closely inspect individual clones for features of XCI and also to examine which form of *MECP2* they express, prior to their use in modeling this disease (Fig 3). Nevertheless, this clonal variability could also pose an advantage for such research, as it enables the isolation of both normal and mutated cell lines from the same patient, which serve as an optimal isogenic system to study the function of *MECP2* in their differentiated neurons (Cheung *et al*, 2011). Moreover, since XCI in hiPSCs-derived neurons retains skewed XCI, these isogenic clones could potentially be mixed together to artificially mimic a "random" XCI pattern that more closely resembles the state in RTT patients. Nevertheless, another study that generated RTT hiPSCs was not able to obtain isogenic lines with reciprocal XCI, but rather reported that all clones reprogrammed from the same parental fibroblasts always inactivated the same X chromosome. However, this was not dependent on *MECP2* mutation, but rather due to rapid selection, occurring either in the fibroblast culture or during reprogramming. Notably, this skewing could be avoided by using an early passage of the fibroblast culture, or by immortalizing them with induced expression of telomerase (Pomp *et al*, 2011). These examples signify the importance in evaluating skewed XCI in hiPSCs, as it directly implicates also the study of additional X-linked disorders in females, such as fragile X (both pre- and full-mutation) and oral–facial–digital syndrome type I (OFD; Franco & Ballabio, 2006).

Apart from skewing, erosion of XCI in hiPSCs might be even more detrimental for modeling X-linked disorders, since it leads to the reactivation of some genes which persists also in the differentiated cells (Fig 3). The effect of this aberration was demonstrated in hiPSCs of Lesch–Nyhan syndrome (LNS) female carriers, which have heterozygous loss-of-function mutations in the gene *HPRT*. This study confirmed that following extended time in culture, female LNS hiPSCs ($Xa^{HPRT-}Xi^{HPRT+}$) became eroded ($Xa^{HPRT-}Xe^{HPRT+}$), resulting in the expression of the wild-type *HPRT*, which was also present in the differentiated neurons. Critically, these later-passage hiPSCs had rescued the disease-related phenotypes of defected neurons, which were apparent in the same cells at lower passages (Mekhoubad *et al*, 2012). This pivotal work highlights the adverse consequences of XCI erosion on the feasibility of female hPSCs to model additional diseases, as erosion of XCI was shown to affect various regions, especially at the distal ends of the X chromosome (Bruck & Benvenisty, 2011). Since *MECP2* is included within these regions, future studies are required to determine whether such erosion indeed leads to its de-repression in hiPSCs of later passage, which will thus hinder the study of RTT in such cells (Mekhoubad *et al*, 2012). Furthermore, the influence of XCI aberrations on disease modeling might extend beyond X-linked disorders, as the extensive upregulation of many X-linked genes following erosion could possibly prompt indirect regulation of downstream autosomal targets. Therefore, these regulatory alterations may potentially interfere with additional phenotypes that could be

involved in other syndromes, or alternatively alter cellular responses toward screened drugs.

Cell therapy

Implementing hPSCs as a source for cellular therapy is a most promising clinical application of these cells. Recent advancements in differentiation protocols toward multiple cell types, along with transplantation experiments in rodents and monkeys, are paving the way toward utilizing hPSCs for treating various conditions, including diabetes, heart diseases, vision loss, spinal cord injury, and Parkinson disease. Excitingly, several clinical trials are currently ongoing, aiming at deciphering the safety and efficiency of hPSC-based therapies (Trounson & DeWitt, 2016). Nevertheless, such treatments raise many concerns as to the effect of potential abnormalities acquired by hPSCs (Ben-David & Benvenisty, 2011). Chromosomal aberrations are generally accepted as serious hazards due to their well-studied involvement in many cancers. Therefore, these genetic changes are inspected and hPSCs that carry such alterations are usually precluded from clinical use. However, epigenetic aberrations are not always as broadly acknowledged and are not routinely tested. Although such changes are also found to be highly prevalent in many tumors, their functional role in driving cancer transformation is still under extensive investigation (Dawson & Kouzarides, 2012).

DNA methylation aberrations are frequently observed in cancer and mostly involve genome-wide hypomethylation, as seen by global reduction in 5-mC levels leading to chromosomal instability and broad transcriptional changes (Eden *et al*, 2003; Ehrlich, 2009; Hansen *et al*, 2011; Peltomäki, 2012). Additionally, hypermethylation in promoters of tumor suppressors is also highly abundant in many cancers (Peltomäki, 2012), and demethylation of CpG islands in promoters of several oncogenes was also reported (Feinberg & Vogelstein, 1983; Van Tongelen *et al*, 2017). These methylation abnormalities confer growth advantage to tumors and enhance their survival. Similar methylation abnormalities are found also in hPSCs and persist in their differentiated counterparts, thus raising concerns regarding potential stimulation of tumorigenic features following their transplantation (Doi *et al*, 2009; International Stem Cell Initiative *et al*, 2011). As of now, growth-related methylation aberrations in hPSCs were found to increase methylation at some promoters, which causes the repression of genes with putative tumor suppressive roles (Fig 3). These findings signify critical ramifications of 5-mC alterations in the safety of hPSCs-based cell therapy, which need to be examined by further studies.

LOI is found to be widespread in many cancers (Jelinic & Shaw, 2007; Uribe-Lewis *et al*, 2011; Kim *et al*, 2015) as well as in various immortalized fibroblasts (Okamura *et al*, 2011). The exact role of this phenomenon and whether it enhances tumor formation is still under investigation, but accumulating evidence suggests that both silencing and overexpression of several imprinted genes are important for tumor growth (Uribe-Lewis *et al*, 2011). This notion is supported by the high frequency of certain cancers in several imprinting disorders. Specifically, patients of Beckwith–Wiedemann syndrome exhibit an early onset of several cancers including Wilm's tumor, hepatoblastoma, rhabdomyosarcoma, adrenocortical carcinoma, and neuroblastoma (Lim & Maher, 2010). In addition, a survey involving patients of Prader–Willi syndrome suggested an increased risk for developing myeloid leukemia (Davies *et al*, 2003). Furthermore, global LOI was demonstrated to drive tumor formation in mice (Holm *et al*, 2005). In humans, hypermethylation of the H19/IGF2 DMR has been associated with additional neoplasia other than those frequent in Beckwith–Wiedemann patients, such as colorectal cancer, chronic myeloid leukemia, and ovarian tumor (Jelinic & Shaw, 2007). Hypermethylation of *MEG3*-DMR or IG-DMR was found in neuroblastomas, pheochromocytoma, Wilm's tumor, and nonfunctioning pituitary adenomas (Astuti *et al*, 2005; Zhao *et al*, 2005; Gejman *et al*, 2008). Notably, many genes that exhibit frequent LOI are shared between tumors and hPSCs. Moreover, the function of some imprinted genes is known to enhance cellular proliferation, reduce apoptosis, and increase pluripotency, which are common features in both cell types. However, not all imprinted genes seem to have a driving role in cancer, thus a close inspection of the regions affected by LOI is necessary to evaluate the safety of these cells for therapy. In addition, since hESCs mostly maintain normal imprinting, they might serve as a better source for applicable transplantations.

Abnormalities associated with the inactive X chromosome have been recurrently described in different cancers. First reported more than 50 years ago, breast and ovarian cancers tend to lose the Barr body, which represents the compacted Xi (Barr & Moore, 1957). This has been attributed to either Xi reactivation or its physical removal along with duplication of the active X (Kawakami *et al*, 2004; Richardson *et al*, 2006; Chaligné & Heard, 2014). Another indication for Xi instability was demonstrated by a global analysis of various cancers, which found that the Xi acquires significantly more somatic mutations in tumors than any other chromosome (Jäger *et al*, 2013). Moreover, a possible regulatory link between *BRCA1* and *XIST* has been suggested by various studies, while others demonstrated conflicting results (Pageau *et al*, 2007). In addition, deleting *Xist* in hematopoietic cells led to the formation of cancer in female mice (Yildirim *et al*, 2013). While additional studies are required to fully understand the effect of XCI aberrations in cancer, the tendency to lose *XIST* expression and reactivate X-linked genes is common to tumors and hPSCs, but very distinct from normal somatic cells. Erosion of XCI in female hPSCs was directly associated with upregulation of cancer-related genes (Fig 3). These include *MAGEA2* and *MAGEA6*, which are X-linked and thus their overexpression is most likely due to partial reactivation of Xi. Furthermore, some cancer autosomal genes were also consistently overexpressed, including *RAB6B* and *ACP5* (Anguera *et al*, 2012), whereas downregulations of autosomal tumor suppressors were highly correlated with *XIST* expression. Apart from inducing transcriptional changes, erosion was also shown to inhibit differentiation and increase proliferation of hPSCs (Anguera *et al*, 2012). Therefore, it seems that promoting cancer development could be an undesirable consequence of XCI erosion. Overall, these findings highlight the potential imperil of employing female hPSCs with XCI erosion as a source for therapeutic transplantations. Male hPSCs or female hiPSCs of early passage, which usually retain XCI, could serve as putative safer alternatives for cell therapy.

Conclusion

Culture-induced aberrations pose a significant concern for utilizing hPSCs in research and in the clinic. Genetic abnormalities, especially chromosomal aberrations, have been thoroughly investigated

and are suggested to affect gene expression and alter the tumorigenicity and differentiation capacity of hPSCs. In recent years, understanding the involvement of epigenetic changes in this regard is gaining more interest. Several epigenetic abnormalities have been frequently reported in hPSCs and include DNA methylation aberrations, loss of parental imprinting, and erosion of XCI. The mechanisms inducing or favoring these alterations are mostly not well understood, and further research aiming at deciphering such driving forces could assist in amending hPSCs culture conditions, as to prevent accumulation of epigenetic aberrations.

Evaluating the influence of epigenetic aberrations on different hPSC-based applications is of great importance. DNA methylation abnormalities might alter hPSC differentiation and therefore affect studies on human development. Moreover, hPSCs with aberrations in genes that are implicated in imprinting disorders are likely unsuitable for modeling other diseases. Finally, further studies are required to better understand the impact of changes in cancer-related genes caused by the different types of epigenetic aberrations. This effect is critical for addressing safety concerns in hPSC-based cellular therapies. Minimizing epigenetic aberrations and their potential adverse effects on the utility of hPSCs is feasible and context-dependent. For example, employing hESCs would be preferable for avoiding LOI, whereas the use of hiPSCs is recommended for preventing erosion of XCI. Also, hESCs exhibit fewer methylation abnormalities at earlier passages, whereas hiPSCs should be stabilized in culture to reduce somatic epigenetic memory. Accordingly, it is important to screen for hPSCs that are largely free of epigenetic alterations, although in many cases, some degree of carefully monitored specific alterations may be tolerated.

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Conflict of interest

The authors declare that they have no conflict of interest.

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